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ORIGINAL ARTICLE

A New Species of the Genus *Xenophrys* (Amphibia: Anura: Megophryidae) from Libo County, Guizhou, China

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Abstract A new species of the genus *Xenophrys* is described from a karst cave environment of Libo County, Guizhou Province, Southern China. The new species, *Xenophrys liboensis* sp. nov., is distinguished from its congeners by a combination of the following characters: 1) tympanum distinct; 2) vomerine teeth present; 3) the length of lower arm and hand larger than the half of snout-vent; 4) heels overlapped slightly when the flexed legs are held at right angles to the body axis; 5) toe tips with rudimentary webs and without grooves; 6) dermal fringes moderate; 7) tubercles on the dorsum forming an X-shaped weak ridge; 8) horn-like tubercle at the edge of the upper eyelid distinct; 9) color of the iris in life is brown. In Bayesian phylogenetic analysis of 22 species of *Xenophrys*, all the individuals of *X. liboensis* sp. nov. clustered into a monophyletic clade with high posterior probabilities. In addition, the ranges of genetic divergences of *X. liboensis* sp. nov. with other species were interspecific rather than intraspecific. Based on the above evidences, we consider that *X. liboensis* sp. nov. is a valid species in *Xenophrys*.

Keywords Megophryidae, *Xenophrys*, new species, phylogeny, southern China

1. Introduction

The *Xenophrys* Günther, 1864 is a genus of amphibians in the Megophryidae family, which is distributed in Southeast Asia, from the southern and eastern Himalayan regions to Borneo (Frost, 2016). Currently, forty-eight species had been described in the genus *Xenophrys*, among which 33 species were recognized from China (Wang *et al*., 2012; Wang *et al*., 2014; Frost, 2016). However, due to the morphological similarity, the horned toads, *Xenophrys*, are still an exemplary group with high cryptic species diversity (Mo *et al*., 2010; Wang *et al*., 2012), making their systematics and taxonomies poorly understood and considerably debated, in spite of various taxonomic methods had been employed (Rao and Yang, 1997; Delorme *et al*., 2006; Li and Wang, 2008; Fei *et al*., 2009; Mahony, 2011). Most cryptic congeners in the genus *Xenophrys* are difficult to be distinguished from each other due to the superficial similarities in morphological features: drab colorations, complicated markings, changeable colorations and skin markings of the same individual under different environmental conditions (Fei *et al*., 2009; Wang *et al*., 2012). Therefore, maybe some cryptic species existed but were covered up by similar/undistinguishable morphological characters (Stuart *et al*., 2006; Pfenninger and Schwenk, 2007). In recent years, a growing number of studies, based on molecular data, corrected the defects of previous morphological research (Li *et al*., 2012; Li *et al*., 2013).

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The genetic data and the phylogenetic reconstructions provided a new opportunity to probe the cryptic species (Rannala and Yang, 2003; Newman *et al*., 2012; Sales *et al*., 2013).

During herpetological surveys conducted from 2015 to 2016 on Libo County (25.4731°N 108.1054°E), Guizhou Province, we found an unknown, relatively big (i.e. body length longer than 50 mm) species which might be assigned into genus *Xenophrys*. Morphologically, these specimens most closely resemble *X. jingdongensis* and *X. omeimontis* (Fei *et al*., 2010, 2012), but differ from *X. minor*, *X. shuichengensis* and *X. spinata*, which distribute in Guizhou and other adjoining provinces. Herein, we describe this population as a new species of *Xenophrys* based on morphological and molecular data.

2. Materials and Methods

2.1 Morphological analyses In this study, morphological analysis was conducted on the new species of living using digital calipers to the nearest 0.1 mm. The measurements and their abbreviations include: SVL (snout-vent length, from tip of snout to vent); HL (head length, from posterior corner of mandible to tip of snout); HW (head width, at the greatest cranial width); SL (snout length, from tip of snout to the anterior corner of eye distance); IN (internarial distance); ED (horizontal eye diameter); IO (interorbital distance, least distance between upper eyelids); UE (upper eyelid width, maximum width of upper eyelid); HTD (horizontal tympanic diameter); LAHL (length of lower arm and hand, from tip of disk of finger III to elbow joint); HAL (hand length, from proximal end of outer palmar tubercle to tip of the third finger); LAD (diameter of lower arm); HLL (hindlimb length or leg length, from tip of disk of toe IV to groin); TL (tibia length with the hindlims flexed); TW (tibia width, the greatest width of tibia); FOL (foot length, from proximal end of inner metatarsal tubercle to tip of fourth toe) and TFL (length of tarsus and foot, from proximal end of tarsus to tip of the fourth toe). We followed the system of description of toe-webbing states used by Savage (1975).

2.2 Molecular analyses

2.2.1 Sample collection During August, 2015 and April, 2016, 13 specimens of *Xenophrys* were collected from Libo County, Guizhou Province, China (Figure 1). Those specimens were euthanized and fixed in 10% formalin and subsequently transferred to 75% ethanol for storage. Before being fixed in formalin, muscle tissues from all

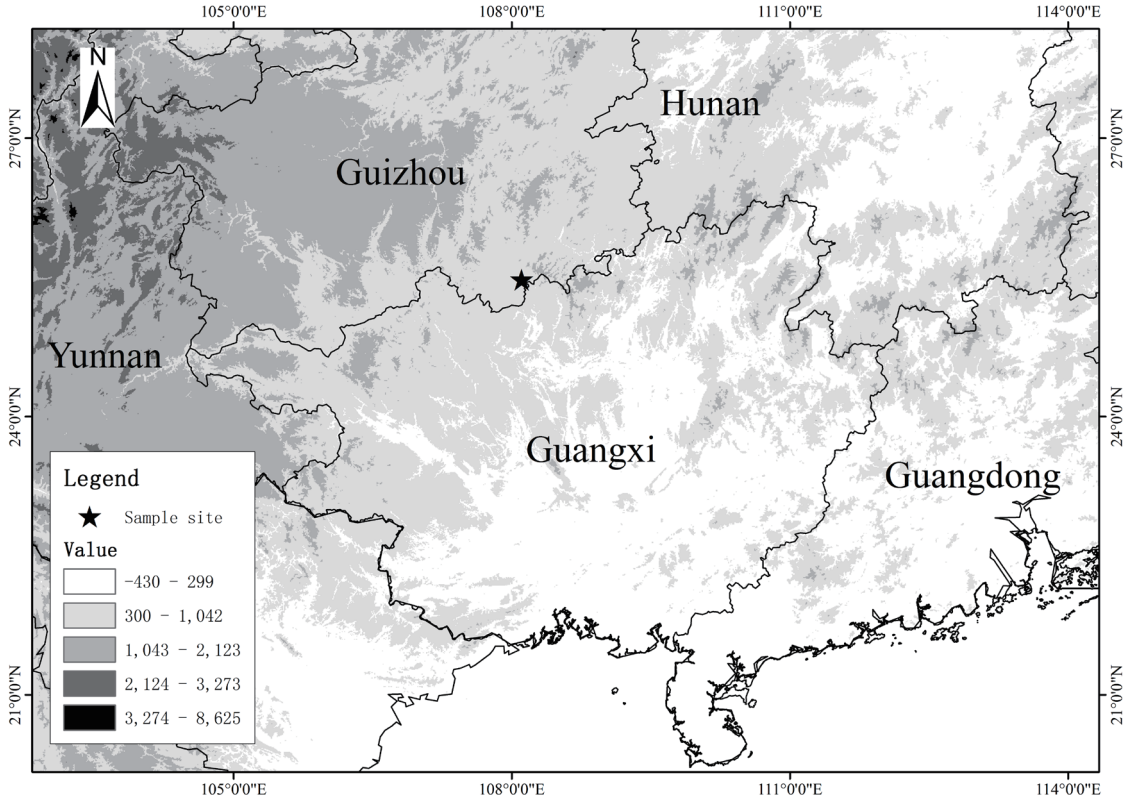

Figure 1 Sampled site of *Xenophrys liboensis* sp. nov. in Guizhou Province.

individuals were sampled and preserved in 100% ethanol for DNA extraction. All specimens and tissue samples were deposited in the Animal Specimen Room of the School of Life Sciences, Guizhou Normal University (GNUG).

2.2.2 DNA Extraction, PCR Amplification, and Sequencing Genomic DNAs were extracted from 6 samples using a standard proteinase K/phenol-chloroform protocol (Sambrook *et al*., 1989). Based on 12S and 16S rRNA gene sequence of *X. omeimontis* (KP728257) and *Leptolalax oshanensis* (KC460337), two pairs of primers (Table 1) were designed to amplify the homologous region using Primer Premier 5.0 (Clarke and Warwick, 2001). PCR were performed using a reaction mixture (25 μL) including 1 μL genomic DNA (concentration 10–50 ng/μL), 2.5 μL 10×buffer, 1 μL of 2.5 mmol/L MgSO4, 2 μL of 2 mmol/L dNTPs, 1 U *Taq* DNA Polymerase (TransGene, China) and 0.3 mmol/L of each of the primer. Pure molecular biology grade water was added to reach the appropriate volume. The amplicon protocol included an initial denaturation step of 95°C for 5 min; this was followed by 32 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and an extension at 72°C for 80 s, with a final extension at 72°C for 10 min. PCR products were purified using an EasyPure PCR Purification Kit (TransGene), and sequenced directly using previous primers and the BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions on an ABI Prism 3730 automated sequencer. All DNA sequences obtained in this study were aligned automatically using Clustal X version 1.83 (Thompson *et al*., 1997), trimmed the length of the fragments and deposited in GenBank (Table S1).

2.2.3 Data collection In present study, homologous sequences of the genus *Xenophrys* available were downloaded from GenBank for phylogenetic analyses, with *Pelobates cultripes*, *Rhinophrynus dorsalis*, *Scaphiopus couchii* and *Spea bombifrons* as the outgroups. In total, 136 sequences were used in our dataset, they involving 87 individuals of 26 species (Table S1).

2.2.4 Phylogenetic analyses Before reconstructing the phylogenetic trees, sequence alignment was carried out using Clustal X 1.8 software with default parameters (Thompson *et al*., 1997), followed by manual adjustment. Nucleotide sites with ambiguous alignments were removed from the analyses, and gaps were analyzed as missing data. The best fit model of evolution (GTR+I+G) was calculated by MrModeltest 1.0 b under the AIC criterion (Nylander, 2003). Bayesian inference of phylogeny was performed using the MrBayes 3.1.2 software program (Huelsenbeck and Ronquist, 2005), with the best fit substitution model. MrBayes analyses simultaneously initiate two Markov Chain Monte Carlo (MCMC) model runs to provide additional confirmation of convergence of posterior probability distributions. Analyses were run for 10,000,000 generations. Chains were sampled every 1000 generations. The first 10% of the total trees were discarded as ''burn-in'' and the remaining trees were used to generate a majority-rule consensus tree and calculate Bayesian posterior probabilities.

2.2.5 Genetic distance analyses Based on our 16S rRNA dataset, we calculated the average genetic distance within 22 *Xenophrys* species (Table 3). Uncorrected pairwise sequence divergence was calculated using MEGA version 5 (Tamura *et al*., 2011).

3. Results

3.1 Taxon description

***Xenophrys liboensis* sp. nov.**

Holotype GNUG20160408008, an adult male (Figure 2) from Libo County, Guizhou Province, China (25.4731°N 108.1054°E, elevation 634m a.s.l.), collected by Gang LI and Jian CHEN on 8, April, 2016.

Paratypes Four males, GNUG20150813001, GNUG20160408001, GNUG20160408004, GNUG20160408007, and eight adult females, GNUG20150813002, GNUG20160408002, GNUG20160408003, GNUG20160408005, GNUG20160408006, GNUG20160408009, GNUG20160408010, GNUG20160408011, were collected at the same locality as the holotype.

Etymology This species is named after the locality, Libo County.

Diagnosis *X. liboensis* sp. nov. is assigned to the genus *Xenophry*s on the basis of the following characters: large body-size (males 34.65–67.70 mm SVL and females 60.78–70.57 mm SVL); snout obtusely pointed, protruding well beyond the margin of the lower jaw; canthus rostralis well-developed; maxillary teeth and vomerine teeth present; margin of the tongue notched behind; tympanum distinct; hind limbs elongated and heels overlapped slightly; skin mostly smooth with some distinct tubercles; tubercles on the dorsum forming an X-shaped weak ridge; a horn-like tubercle at the edge of

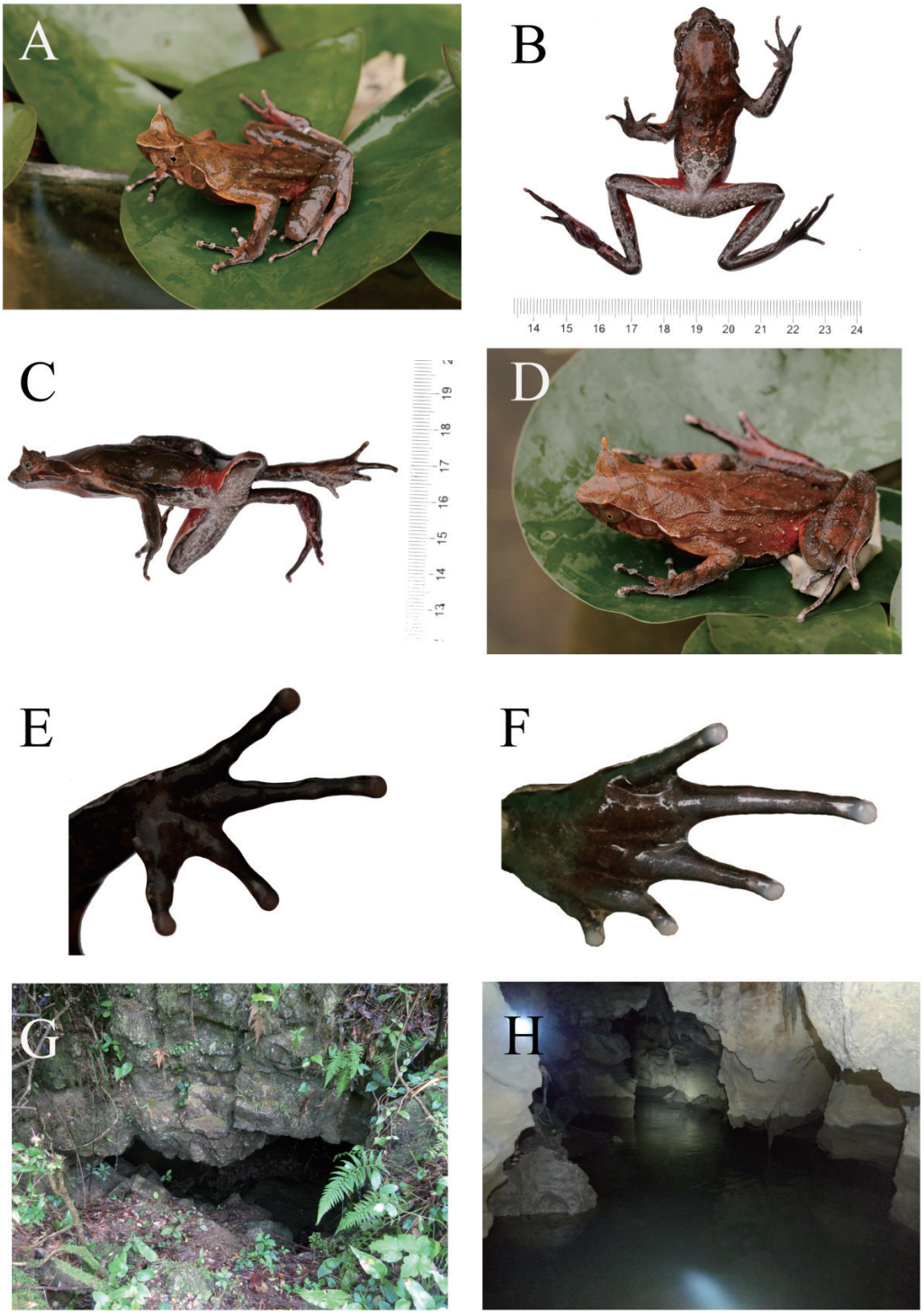

Figure 2 *Xenophrys liboensis* sp. nov. (A) Dorsolateral view of the live adult male *X. liboensis* sp. nov. holotype GNUG20160408008; (B) Ventral view of the live holotype; (C) Lateral view of the live holotype; (D) Dorsolateral view of the live adult female *X. liboensis* sp. nov. paratypes GNUG20160408002; (E) Ventral view of the right hand of the live adult male *X. liboensis* sp. nov. holotype; (F) Ventral view of the right foot of the live adult male *X. liboensis* sp. nov. holotype; (G) and (H) The habitats of *X. liboensis* sp. nov. in Libo County, Guizhou Province, China.

the eyelid distinct; an inverted triangular brown speckle between two upper eyelids; supratympanic fold distinct; pupil vertical; single vocal sac in males.

X. liboensis sp. nov. is distinguished from its congenerous species by the following combination of morphological characters: 1) tympanum distinct; 2) vomerine teeth present; 3) the length of lower arm and hand longer than the half of snout-vent; 4) heels

Table 1 Primers used in PCR amplification and sequenced in this study.

Target gene	Primer	Primer Sequence	Site (bp)	Source
The complete 12S ribosomal gene	Xeno-12S-F	5'- GCTTACCATAAAGCACAGCACTGAAG -3'	1082	This study
	Xeno-12S-R	5'- CACCTTGACCTGACTTACT -3'	1781	This study
The complete 16S ribosomal gene	Xeno-16S-F	5'- GAACTCGGCAAATCAAAGTCCCGCCTG -3'	2988	This study
	Xeno-16S-R	5'- GGCTCTACTTTTTCGGTCCTTTCGTAC -3'	3658	This study

overlapped slightly when the flexed legs are held at right angles to the body axis; 5) toe tips with rudimentary webs (between toe tips I–II; and toe tips II–III) and without circummarginal grooves; 6) dermal fringes moderate; 7) tubercles on the dorsum forming an X-shaped weak ridge; 8) horn-like tubercle at the edge of the upper eyelid distinct; 9) color of the iris in life is brown.

Description of the holotype Adult male (Figure 2), body length 60.53 mm, further measurements listed in Table 2. Body slender; head length slightly shorter than head width (HL/HW = 0.91); snout obtusely pointed, protruding well beyond the margin of the lower jaw; canthus rostralis well-developed, loreal region vertical and oblique slightly; eye large and convex, eye diameter 0.39 of head length; interorbital width larger than upper eyelid width (IO/UE = 1.67); interorbital space flat; pupil vertical; tympanum distinct and oval, larger than half eye width (HTD/ED$^{1}/_{2}$ = 1.20); maxillary teeth present; vomerine teeth present, tongue moderate and oval, with small notch at posterior tip; internal single subgular vocal sac present, openings near the corners of mouth; and supratympanic fold distinct, from eye towards axillary gland.

Forelimbs slender, the length of lower arm and hand larger than the half of snout-vent; fingers slender, without webbing, dermal fringes feeble; relative length of fingers: I < II < IV < III; finger tips rounded, slightly enlarged; subarticular tubercles indistinct, rounded and slightly protuberant; inner palmar tubercle moderate and elliptical, outer palmar tubercle small and flat.

Hindlimbs slender (TBL/SVL = 0.51), tibiotarsal articulation reaching to the middle of eye when limbs adpressed anteriorly, heels overlapped slightly when the flexed legs are held at right angles to the body axis; foot shorter than shank slightly (FOL/TBL = 0.90); toe tips rounded, slightly enlarged, without circummarginal grooves, with rudimentary webs; dermal fringes moderate; relative toes length I < II < V < III <IV ; subarticular tubercles present but with indistinct edges, rounded and slightly protuberant; and inner metatarsal tubercle small and elliptical, and outer metatarsal tubercle absent.

Skin mostly smooth, with distinct tubercles on the dorsal and lateral parts of the body; tubercles on the dorsum forming an X-shaped weak ridge; several tubercles on the flanks and dorsal surface of thighs and tibias and forming four transverse tubercle rows; a horn-like tubercle at the edge of the upper eyelid distinct; an inverted triangular brown speckle between two upper eyelids; supratympanic fold distinct; ventral surface smooth; chest gland small and round, closer to the axilla than to the mid-ventral line; femoral gland on rear of thigh; posterior end of the body protrudes distinct and appears as an arc-shaped swelling, upper the anal region.

Coloration of holotype in life Rust above with maroon markings, including a triangular marking bordered with a vermilion edge between the eyes, apex of triangle over occiput, an X-shaped marking on the dorsum of body, four rosewood transverse bands on the dorsal surface of the thigh and shank separately; several rosewood vertical bars on the lower and upper lip; supratympanic fold white; posterior surface of the flank and anterior surface of the thighs near the groin red; a black streak on the ventrolateral regions distinct; throat, chest carmine and belly grey all with rosewood blotches; chest and femoral glands white; ventral surface of limbs grey with many tiny white spots; iris brown with vertical black pupil.

Coloration of holotype in preservative Dorsal surface saddle brown with seal brown markings; triangular and X-shaped markings on dorsum and transverse bands on limbs distinct; throat, chest saddle brown and belly grey with seal brown blotches; black streak distinct; peach-orange substitutes the red in the anterior surface of the thighs and lateral surface of the trunk.

Variation Variation in measurements given in Table 2. Most females larger than males, SVL 60.78–70.57 mm in females (n = 8) and 34.65–67.70 mm in males (n = 5). Individuals relatively uniform in body coloration. The sample GNUG20150813001 (one of paratypes) is a sub-adult, smaller than others.

Secondary sexual characters An internal single subgular vocal sac is present in males, with vocal sac openings near the corners of the mouth.

Distribution This species is known only in Libo County,

Table 2 Measurements (in mm) of *Xenophrys liboensis* sp. nov., collected from Libo County, Guizhou Province, China.

	Xenophrys liboensis sp. nov.												
	Holotype	Paratypes											
		Males				Females							
	GNUG 20160408008	GNUG 20160408001	GNUG 20160408004	GNUG 20160408007	GNUG 20150813001	GNUG 20160408002	GNUG 20160408003	GNUG 20160408005	GNUG 20160408006	GNUG 20160408009	GNUG 20160408010	GNUG 20160408011	GNUG 20150813002
SVL	60.53	61.55	67.7	62.94	34.65	62.84	60.78	62.91	64.71	70.57	65.17	61.18	65.22
HL	18.38	19.85	18.65	18.68	11.77	18.22	17.18	19.93	18.15	19.7	19.5	19.03	18.1
HW	20.23	21.28	20.95	20.56	11.75	21.05	20.42	20.84	20.49	21.65	21.12	20.44	20.01
SL	7.41	7.04	7.64	7.45	2.66	7.59	7.42	7.33	7.17	7.4	8.36	6.17	7.42
IN	6.22	6.08	6.91	6.64	4.44	5.99	6.71	6.29	6.84	6.93	7.21	7.1	7.07
ED	7.22	7	7.05	7.35	4.8	8.07	7.42	7.43	7.74	6.46	7.53	7.28	8.82
IO	5.73	5.41	5.67	5.65	2.98	5.62	5.5	5.63	5.8	5.95	6.03	5.58	6.15
UE	3.43	3.75	4.62	3.77	3.4	4.07	4.8	4.37	3.67	4.47	4.67	5.11	4.55
HTD	4.33	4.28	4.37	4.49	2.3	5.02	4.71	4.84	4.62	5.01	4.31	4.74	4.73
LAHL	33.54	31.74	36.06	33.86	15.85	32.04	34.01	32.89	32.7	33.65	35.18	33.45	33.01
HAL	18.59	17.81	18.75	17.64	8.75	18.12	17.57	17.97	17.87	19.12	18.99	17.78	17.38
LAD	5.87	5.58	5	5.81	4.6	5.86	5.4	5.79	5.45	5.6	5.15	5.34	5.1
HLL	95.95	92.68	99.17	92.49	53.58	96.66	98.14	91.99	93.83	99.42	96.19	94.45	94.81
TL	31	29.85	32.31	30.22	17.58	30.58	31.08	29.49	28.28	30.98	32.6	29.21	29.14
TW	6.45	5.74	6.12	5.98	3.99	6.09	5.83	6.21	6.18	6.59	5.75	6.4	5.91
FOL	28.01	27.94	29.4	25.15	15.75	27.66	30.12	27.05	28.09	29.73	29.66	30.17	27.23
TFL	43.71	44.49	46.75	41.31	23.58	43.16	43.21	41.83	41.45	45.12	45.4	42.98	43.48

Guizhou Province, China.

Ecological notes A total of 13 individuals, including adults and sub-adult were founded on the rock near a approximate 2×60 m^2 pool (Figure 2 H), about 35 m from the entrance of the cave. The depth of water was about 50 cm, the water temperature was approximate 10°C, and the air temperature was approximate 15°C in the cave, tested in April. The cave surrounded by evergreen broadleaved forests and broadleaved deciduous forest at elevations of about 634 m. The collecting site was in darkness. Outside the cave, no adults and tadpoles of the new species were found during a herpetological survey in the vicinity.

3.2 Comparisons We compared the new adult species of the *Xenophrys* with their 32 known congeners which distribute in China.

Geographically, the distributions of *X. liboensis* sp. nov., *X. minor*, *X. shuichengensis* and *X. spinata* closely. However, *X. liboensis* sp. nov. differs from *X. minor* by the presence of vomerine teeth (vomerine teeth absent in *X. minor*), horn-like tubercle at the edge of the upper eyelid distinct (the tubercle of *X. minor* indistinct), relative length of finger, I < II < IV < III (*vs*. II = I = IV < III in *X. minor*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. shuichengensis* by the body-size smaller relatively (males 34.65–67.70 mm SVL and females 60.78–70.57 mm SVL for *X. liboensis* sp. nov. *vs*. males 100–116 mm SVL and females 102–118 mm SVL for *X. shuichengensis*), horn-like tubercle at the edge of the upper eyelid distinct (*vs.* indistinct), relative length of finger, I < II < IV < III (*vs*. II = I = IV < III in *X. shuichengensis*), toe tips with rudimentary webs (one third web of toe IV in *X. shuichengensis*) and dermal fringes moderate (dermal fringes developed in *X. shuichengensis*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. spinata* by the length of lower arm and hand longer than the half of snout-vent (the length of lower arm and hand shorter than the half of snout-vent in *X. spinata*), the dermal fringes moderate (dermal fringes wide and distinct in *X. spinata*) and toe tips with rudimentary webs (toe tips with half webs in *X. spinata*) (Fei *et al.*, 2010, 2012).

X. liboensis sp. nov. is morphologically similar to *X. caudoprocta* and *X. pachyproctus*. But, *X. liboensis* sp. nov. differs from *X. caudoprocta* by the presence of vocal sac openings (vocal sac openings absent in *X. caudoprocta*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. pachyproctus* by the body-size bigger relatively (males 34.65–67.70 mm SVL and females 60.78–70.57 mm SVL for *X. liboensis* sp. nov. *vs*. males 35–36 mm SVL and females 36 mm SVL for *X. pachyproctus*) and toe tips with rudimentary webs (toe tips without webs for *X. pachyproctus*) (Fei *et al.*, 2010, 2012).

The genetic relationship of *X. liboensis* sp. nov., *X. huangshanensis*, *X. boettgeri*, *X. jingdongensis*, *X. omeimontis X. spinata*, *X. kuatunensis*, *X. cheni*, *X. lini*, *X. brachykolos* and *X. jinggangensis* are closer (Figure 3, Table 3). Besides the *X. spinata* which compared above, *X. liboensis* sp. nov. differs from *X. huangshanensis* by tympanum distinct (tympanum indistinct for *X. huangshanensis*), the length of lower arm and hand longer than the half of snout-vent (the length of lower arm and hand shorter than the half of snout-vent for *X. huangshanensis*), and heels overlapped slightly when the flexed legs are held at right angles to the body axis (heels separated in *X. huangshanensis*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. boettgeri* by the presence of vomerine teeth (vomerine teeth absent in *X. boettgeri*), tubercles on the dorsum forming an X-shaped weak ridge (*X. boettgeri* lack the marking) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. jingdongensis* by the tubercles on the dorsum forming an X-shaped weak ridge which in *X. jingdongensis* is Y-shape, the length of lower arm and hand longer than the half of snout-vent (the length of lower arm and hand shorter than the half of snout-vent for *X. jingdongensis*), toe tips with rudimentary webs (toe tips with half webs for *X. jingdongensis*) and subarticular tubercles present of toe tips (subarticular tubercles present of toe tips absent in *X. jingdongensis*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. omeimontis* by the different color of the iris in life (brown *vs*. orange in the upper half and brown in the lower half), and belly grey in life (belly buff for *X. omeimontis*), relative length of finger I < II < IV < III (IV = I < II < III in *X. omeimontis*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. kuatunensis* by the body-size bigger relatively (males 34.65–67.70 mm SVL and females 60.78–70.57 mm SVL for *X. liboensis* sp. nov. *vs*. males 26–30 mm SVL and females 37 mm SVL for *X. kuatunensis*), presence of vomerine teeth (vomerine teeth absent in *X. kuatunensis*) and heels overlapped slightly when the flexed legs are held at right angles to the body axis (heels separated in *X. kuatunensis*) (Fei *et al.*, 2010, 2012). *X. liboensis* sp. nov. differs from *X. cheni* and *X. lini* by the body-size bigger relatively (body length of all adults >60 mm for new species *vs.* body length of all adults <40 mm for *X. cheni* and *X. lini*), presence of vomerine teeth (vomerine teeth absent in *X. cheni* and *X. lini*) and tongue feebly notched (tongue deeply notched in *X. cheni* and not notched in *X. lini*) (Wang *et al*., 2014). *X. liboensis* sp. nov. differs

Table 3 Uncorrected pairwise sequence divergence estimates (%) among 22 *Xenophrys* species in this study, based on 16S rRNA gene sequence.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*X. shapingensis*																						
2	*X. mangshanensis*	9.8																					
3	*X. kuatunensis*	9.7	11																				
4	*X. boettgeri*	9.4	11.3	3.4																			
5	*X. huangshanensis*	10.9	12.3	3.4	0.5																		
6	*X. minor*	9.3	12.6	6.8	5.6	6.4																	
7	*X. cheni*	8.8	11.6	2.8	3.3	3.3	6.4																
8	*X. jinggangensis*	8.3	11.9	4.7	4.2	4.5	6.5	3.2															
9	*X. brachykolos*	10.5	13	5.3	6.2	6.3	8.1	5.5	6.1														
10	*X. lini*	9.4	12.1	3.9	4.5	4.6	7.7	3.8	4.4	5.1													
11	*X. major*	8.5	9.8	12.6	12.9	14.4	12.9	11.8	11.1	13.5	12.3												
12	*X. baluensis*	11.6	15.2	15.6	15.6	16.5	16	15.5	14.9	14.7	15	15.1											
13	*X. omeimontis*	7.9	10.9	4.9	5	4.9	7.3	4.5	5.7	7.3	5.6	12.1	14.4										
14	*X. nankiangensis*	1.5	11	10.3	10.7	11.5	10.4	10	9.6	11.6	10.1	9.1	12.2	8.9									
15	*X. maosonensis*	10.9	2.6	13	14.2	14.5	15.8	14.5	14.6	16.2	15	9.1	18.6	11.7	11.7								
16	*X. jingdongensis*	9.6	13	7.5	7.1	6.6	9.9	6.2	7	11	8.3	13.5	18.1	3.3	10.7	12.1							
17	*X. dringi*	8.1	13.4	12.3	11.8	12.9	11.7	12.6	12.9	13.1	11.8	11.4	13.2	11.2	9	14.8	14						
18	*X. parva*	8.5	10.5	7.7	5.4	6.6	7.7	6.3	6.3	8.4	6.6	14.4	14.6	4.7	9.9	13.4	4.8	11.7					
19	*X. aceras*	10	13	14.7	13	14.5	13.4	12.6	11.7	14.8	13.6	12.4	16.8	11.2	12.3	14.9	13.2	14	12.7				
20	*X. spinata*	8.6	11.7	5.8	4.9	4.9	7.7	4	5.3	8.7	5.9	12.8	14.9	3	9.2	14.1	3.5	12.6	6.3	12.3			
21	*X. legkaguli*	2.3	8.2	5.1	7.4	6	6.8	6.8	6.9	8.1	5.3	3	8	3.5	1.7	5.4	5.4	6.6	7.5	6	3.4		
22	*X. liboensis* sp. nov.	8.7	11.5	3.8	2.6	2.3	6.2	4.5	5.3	6.2	5.6	12.1	15.1	5.2	10.7	14.2	7.4	11.6	6.6	12.8	6.2	4.7	

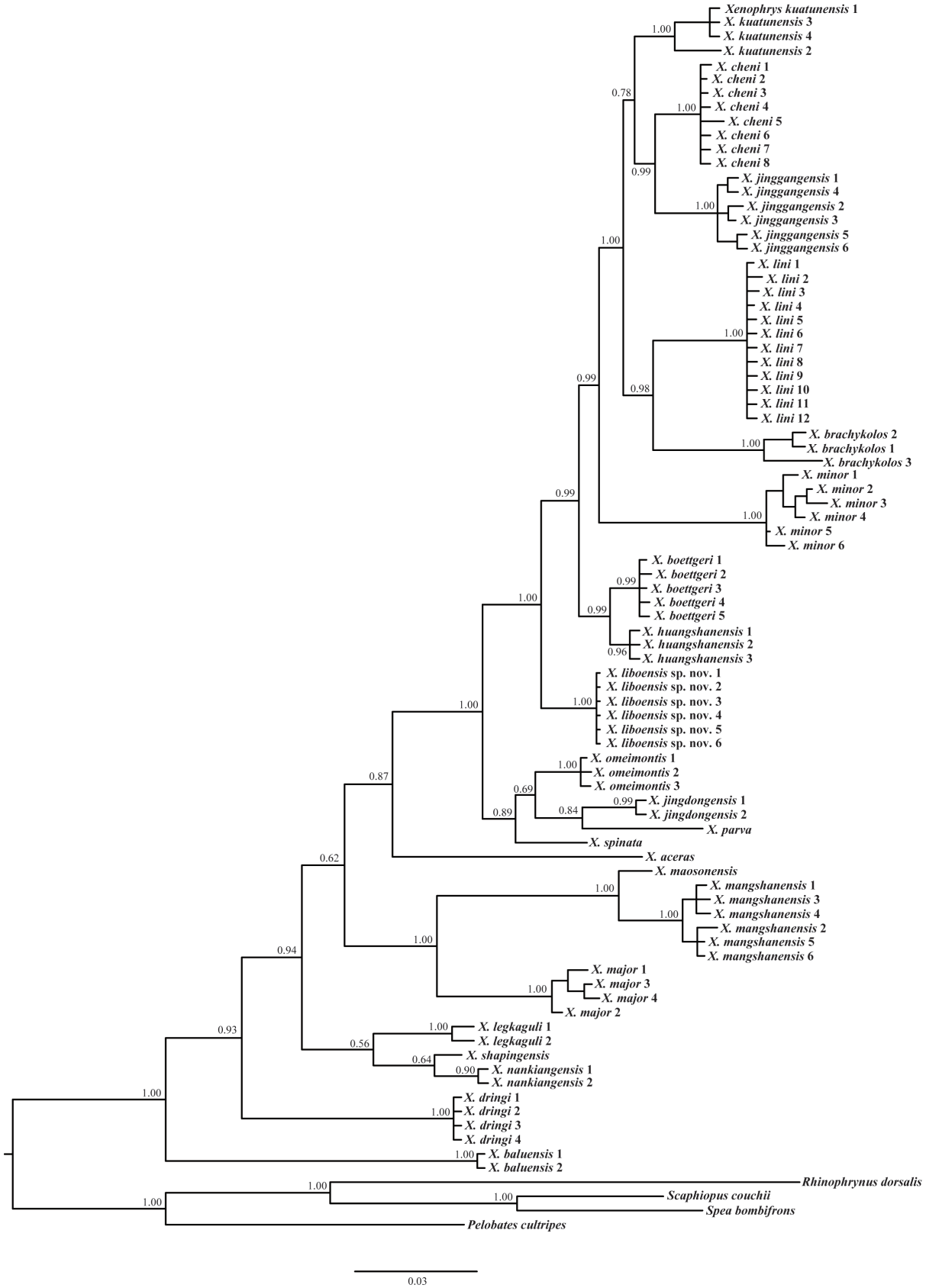

Figure 3 Bayesian phylogenetic tree of 22 species in genus *Xenophrys*, based on the combined mitochondrial dataset. The numbers near branches were posterior probabilities. Four species, *Pelobates cultripes*, *Rhinophrynus dorsalis*, *Scaphiopus couchii* and *Spea bombifrons*, were employed as outgroup in phylogeny analysis.

from *X. brachykolos* by the body-size bigger relatively (body length of all adults >60 mm for new species *vs.* body length of all adults <46 mm in *X. brachykolos*), presence of vomerine teeth (vomerine teeth absent in *X. brachykolos*), heels overlapped slightly when the flexed legs are held at right angles to the body axis (heels separated in *X. brachykolos*) and relative length of finger I < II < IV < III (*vs*. IV < II < I < III in *X. brachykolos*) (Fei *et al.*, 2012). *X. liboensis* sp. nov. differs from *X. jinggangensis* by the body-size bigger relatively (body length of all adults >60 mm for new species *vs.* body length of all adults <42 mm in *X. jinggangensis*), tongue feebly notched (tongue not notched in *X. jinggangensis*) and relative length of finger I < II < IV < III (*vs*. II < I < IV < III in *X. jinggangensis*) (Wang *et al*., 2012).

Of the remaining 18 species, 14 of them (*X. acuta*, *X. baolongensis*, *X. binchuanensis*, *X. binlingensis*, *X. daweimontis*, *X.maosonesis*, *X. obesa*, *X. pachyproctus*, *X. palpebralespinosa*, *X. parva*, *X. tuberogranulatus*, *X. wuliangshanensis*, *X. zhangi*, *X. sangzhiensis*) (body length of males <51 mm, body length of females <55 mm) were smaller than the new species (body length of all adults >60 mm) (Mo *et al.*, 2010; Fei *et al.*, 2010, 2012; Wang *et al*., 2012; Wang *et al*., 2014; Li *et al*., 2014). In addition, *X. liboensis* sp. nov. differs from *X. glandulosa*, *X. major*, *X. mangshanensis* and *X. medogensis* by the length of lower arm and hand longer than the half of snout-vent (*vs*. the length of lower arm and hand shorter than the half of snout-vent) (Fei *et al.*, 2010, 2012).

3.3 Molecular analyses

3.3.1 Phylogenetic analyses For each individual of *X. liboensis* sp. nov., the sequence fragments of 1228 base pairs were obtained, including a 595 bp 16S rRNA gene fragment and a 633 bp 12S rRNA gene fragment. In addition, 16S rRNA gene fragments of 77 individuals of 21 *Xenophrys* species, 12S rRNA gene fragments of 29 individuals of 9 *Xenophrys* species, and the homologous fragments of four outgroups, were combined together for the further analysis (Table S1). After sequence alignment, the 16S rRNA gene yielded 211 variable sites and the 12S rRNA gene yielded 227 variable sites. Based on datasets of 12S rRNA and 16S rRNA, the BI phylogenetic approaches obtained a tree with relatively high-supporting values in terminal clades. In the Bayesian phylogenetic tree, twenty-two species of *Xenophrys* formed a distinct monophyletic lineage (Figure 3). Among them, *X. liboensis* sp. nov. constituted a distinct phylogenetic lineage with strong support (1.0 for Bayesian posterior probability; Figure 3), and acted as the sister group to the terminal clade which comprised of 8 *Xenophrys* species (*X. kuatunensis*, *X. cheni*, *X. jinggangensis*, *X. lini*, *X. brachykolos*, *X. minor*, *X. boettgeri* and *X. huangshanensis*). In this clade, the 8 species was generally characterized by the small body size (26–48 mm) (Wang *et al*., 2014).

3.3.2 Genetic distance analyses Among 22 *Xenophrys* species in this study, the uncorrected pairwise sequence divergence ranges from 0.5% (*X. huangshanensis vs*. *X. boettgeri*) to 18.6% (*X. maosonensis vs*. *X. baluensis*) for 16S rRNA gene. If *X. liboensis* sp. nov. is excluded, the genetic distances still maintain above variation range. For *X. liboensis* sp. nov., the genetic distance with other species of *Xenophrys* ranges from 2.3% (*X. liboensis* sp. nov. *vs*. *X. huangshanensis*) to 15.1% (*X. liboensis* sp. nov. *vs*. *X. baluensis*) (Table 3).

4. Discussion

In this study, we discovered an undescribed species of the genus *Xenophrys*, named *X. liboensis* sp. nov. combining morphological characters and molecular data. In the result, all the individuals of *X. liboensis* sp. nov. formed into a distinct monophyletic clade in phylogenetic analysis (Figure 3) with robust support value, as well as genetic divergences, which indicated that *X. liboensis* sp. nov. is a distinct species. The obviously morphological characters were also found between *X. liboensis* sp. nov. and its congeners. On the basis of all the above evidences, we consider that *X. liboensis* sp. nov. is a valid species in *Xenophrys*. Currently, only three species of *Xenophrys* (*X. minor*, *X. shuichengensis* and *X. spinata*) reported in Guizhou Province, the new species represents a new record located Guizhou of the genus *Xenophrys* and its discovery is significant to unveil the cryptic species diversity of *Xenophrys* toads.

Further, *X. liboensis* sp. nov. was only founded in dark karst cave of southeastern Guizhou Province. Considering extensive well-developed karst landscapes in this region, we argue that the *X. liboensis* sp. nov. might range widely in the region. Therefore, more fieldwork needs to be done to determine its distribution range and population size. Furthermore, previous studies had reported that some species of frog, *Odorrana wuchuanensis*, *O. lipuensis* and *Oreolalax rhodostigmatus* also inhabited in environments of dark karst cave (Fei *et al*., 2009, 2012; Mo *et al*., 2015). So it seems that, for some frogs, dark karst cave is utilizable environment, but how the species has adapted to subterranean environments, it need further study in the future.

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Figure S1 Dorsolateral view of the adult male *Xenophrys liboensis* sp. nov. holotype GNUG20160408008 in preservative.

Figure S2 Ventral view of the adult male *Xenophrys liboensis* sp. nov. holotype GNUG20160408008 in preservative.

Table S1 Sampling locations, voucher date, and associated GenBank accession numbers of *Xenophrys* toads in this study.

	Species	No.	Locality	Voucher	GenBank Accession No.	
					16S rRNA	12S rRNA
1	*X. mangshanensis*	1	Unknown Origin	SYSA0000496	JX867335	-
		2	Nanling, Guangdong, China	SYS a001564	KJ560371	-
		3	Nanling, Guangdong, China	SYS a002025	KJ560372	-
		4	Nanling, Guangdong, China	SYS a002026	KJ560373	-
		5	Jiulianshan, Jiangxi, China	SYS a002118	KJ560374	-
		6	Jiulianshan, Jiangxi, China	SYS a002119	KJ560375	-
2	*X. kuatunensis*	1	Wuyishan, Fujian, China	SYS a001579	KJ560376	-
		2	Unknown Origin	SYSA0000241	JX867341	-
		3	Wuyishan, Fujian, China	SYS a001592	KJ560378	-
		4	Wuyishan, Fujian, China	SYS a001590	KJ560377	-
3	*X. boettgeri*	1	Tongbashan, Jiangxi, China	SYS a001700	KJ560382	KJ560419
		2	Tongbashan, Jiangxi, China	SYS a001683	KJ560381	KJ560418
		3	Tongbashan, Jiangxi, China	SYS a001673	KJ560380	KJ560417
		4	Yangjifeng, Jiangxi, China	SYS a000378	KJ560379	-
		5	Wuyishan, Fujian, China	SYSA0000378	JX867340	-
4	*X. huangshanensis*	1	Wuyishan, Fujian, China	SYS a001623	KJ560385	-
		2	Wuyuan, Jiangxi, China	SYS a001622	KJ560384	-
		3	Wuyuan, Jiangxi, China	SYS a001322	KJ560383	-
5	*X. minor*	1	Laojunshan, Sichuan, China	SYS a002166	KJ560390	KJ560424
		2	Laojunshan, Sichuan, China	SYS a002165	KJ560389	KJ560423
		3	Laojunshan, Sichuan, China	SYS a002164	KJ560388	KJ560422
		4	Emeishan, Sichuan, China	SYS a001805	KJ560387	KJ560421
		5	Emeishan, Sichuan, China	SYS a001804	KJ560386	KJ560420
		6	Unknown Origin	ZYC1500	AY561308	-
6	*X. cheni*	1	Taoyuandong, Hunan, China	SYS a002142	KJ560398	KJ560429
		2	Taoyuandong, Hunan, China	SYS a002124	KJ560397	KJ560428
		3	Taoyuandong, Hunan, China	SYS a002123	KJ560396	KJ560427
		4	Jinggangshan, Jiangxi, China	SYS a001872	KJ560395	KJ560426
		5	Jinggangshan, Jiangxi, China	SYS a001871	KJ560394	KJ560425
		6	Jinggangshan, Jiangxi, China	SYS a001429	KJ560393	-
		7	Jinggangshan, Jiangxi, China	SYS a001428	KJ560392	-
		8	Jinggangshan, Jiangxi, China	SYS a001427	KJ560391	-
7	*X. jinggangensis*	1	Taoyuandong, Hunan, China	SYS a002132	KJ560402	KJ560433
		2	Taoyuandong, Hunan, China	SYS a002131	KJ560401	KJ560432
		3	Taoyuandong, Hunan, China	SYS a001860	KJ560400	KJ560431
		4	Taoyuandong, Hunan, China	SYS a001859	KJ560399	KJ560430
		5	Jinggangshan, Jiangxi, China	SYSA0001414	JX867338	-
		6	Jinggangshan, Jiangxi, China	SYSA0001416	JX867337	-
8	*X. brachykolos*	1	Hongkong, China	SYS a002259	KJ560404	KJ560435
		2	Hongkong, China	SYS a002258	KJ560403	KJ560434
		3	Hongkong, China	SYSA0001502	JX867339	-
9	*X. lini*	1	Taoyuandong, Hunan, China	SYS a002128	KJ560416	KJ560441
		2	Bamianshan, Jiangxi, China	SYS a002383	KJ560415	KJ560440
		3	Bamianshan, Jiangxi, China	SYS a002382	KJ560414	KJ560439
		4	Suichuan, Jiangxi, China	SYS a002371	KJ560413	KJ560438
		5	Suichuan, Jiangxi, China	SYS a002370	KJ560412	KJ560437
		6	Jinggangshan, Jiangxi, China	SYS a002380	KJ560411	KJ560436
		7	Jinggangshan, Jiangxi, China	SYS a001491	KJ560410	-
		8	Jinggangshan, Jiangxi, China	SYS a001490	KJ560409	-
		9	Jinggangshan, Jiangxi, China	SYS a001489	KJ560408	-
		10	Jinggangshan, Jiangxi, China	SYS a001422	KJ560407	-
		11	Jinggangshan, Jiangxi, China	SYS a001421	KJ560406	-
		12	Jinggangshan, Jiangxi, China	SYS a001420	KJ560405	-
10	*X. major*	1	Phongsali, Long Nai Khao, Laos	2004.028	KR828085	-
		2	Phitsanulok, Phu Soi Dao waterfall, Thailand	0025Y	KR828084	-
		3	Route 20, km 6, Phong Nha-Ke Bang, National Park, Viet Nam	ZFMK 86361	HQ588953	-
		4	Cha Noi, Phong Nha-Ke Bang National, Park, Viet Nam	ZFMK 86975	HQ588952	-
11	*X. baluensis*	1	Unknown Origin	ZMH A13125	KJ831310	-
		2	Unknown Origin	ZMH A13124	KJ831309	-

Continued Table S1

	Species	No.	Locality	Voucher	GenBank Accession No.	
					16S rRNA	12S rRNA
12	*X. omeimontis*	1	Unknown Origin	MO-HY20130601	KP728257	KP728257
		2	Omei Mt., Sichuan, China	ROM40462	EF397242	EF397242
		3	Unknown Origin	ZYC1513	AY561307	-
13	*X. nankiangensis*	1	Nan Jiang Co., Sichuan, China	CIB-XM835	EF397243	EF397243
		2	Unknown Origin	XM835	AY526200	-
14	*X. maosonensis*		Vinh Phuc, Tam Dao, Viet Nam	2000.2935	KR828086	-
15	*X. jingdongensis*	1	Yunnan, Lushun, China	2001.0278	KR828083	-
		2	Lao Cai, Sapa, Viet Nam	1999.5688	KR828082	-
16	*X. dringi*	1	Unknown Origin	UNIMAS 8943	KJ831317	-
		2	Unknown Origin	UNIMAS 8948	KJ831316	-
		3	Unknown Origin	UNIMAS 8942	KJ831315	-
		4	Unknown Origin	ZMH A09364	KJ831314	-
17	*X. parva*		Sapa, Viet Nam	MNHN:1999.5694	JN848362	-
18	*X. aceras*		West Malaysia, Perak, Temengor Forest, Malaysia	LSUHC_7038	GQ995534	-
19	*X. spinata*		Unknown Origin	ZYC644	AY526205	-
20	*X. legkaguli*	1	Unknown Origin	FMNH 265959	DQ860093	-
		2	Unknown Origin	FMNH 266341	DQ860092	-
21	*X. shapingensis*		Unknown Origin	CIBSC2011102004	JX458090	JX458090
22	*X. liboensis* sp. nov.	1	Libo Country, Guizhou, China	20150813001	MF285253*	MF285242*
		2	Libo Country, Guizhou, China	20150813002	MF285254*	MF285243*
		3	Libo Country, Guizhou, China	20160408004	MF285256*	MF285245*
		4	Libo Country, Guizhou, China	20160408006	MF285257*	MF285246*
		5	Libo Country, Guizhou, China	20160408007	MF285258*	MF285247*
		6	Libo Country, Guizhou, China	20160408009	MF285260*	MF285249*
23	*Pelobates cultripes*		Las Rozas, Madrid, Spain	/	AJ871086	AJ871086
24	*Rhinophrynus dorsalis*		Unknown Origin	MVZ: Herp: 164755	JX564892	JX564892
25	*Scaphiopus couchii*		Unknown Origin	MVZ: Herp: 245863	JX564894	JX564894
26	*Spea bombifrons*		Unknown Origin	MVZ: Herp: 240065	JX564896	JX564896

Note: "-" represens no molecular data; "/" represents the voucher unknown; "*" means that it is new sequence in this study.